



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No. **03-40155-US**

Inventors: Ho et al.

Group Art Unit No.: 1621

Application No.: 10/652,813

Examiner: Witherspoon, Sikarl A.

Filed: August 29, 2003

For: Benzotropolone Derivatives And Modulation Of Inflammatory Response

MS Amendment  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

**DECLARATION UNDER RULE 132**

As below named coinventors of the subject matter claimed in the above-identified application, we do hereby declare that:

1. We along with Robert T. Rosen, who is unable to sign this declaration as he is presently incapacitated and is in an intensive care unit, are coauthors of the research publication in Tetrahedron Letters, 2002, 43:7129-7133.
2. The other authors of the publication are Shiying Tian, Ruth E. Stark, Xiaofeng Meng and Chung S. Yang ("other authors").
3. The other authors merely worked under our direction and were not involved in conception of the invention including the design and methods of synthesis of the benzotropolone derivatives recited in the pending claims. Therefore, the other authors are not co-inventors of the subject matter disclosed in the research publication.

By signing below, we hereby further declare that all statements made herein of our own knowledge are true and that all statements made on information or belief are believed to be true;

and further that these statements were made with the knowledge that willful statements and the like so made are punishable by fine or by imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the application, any patent issuing there upon, or any patent to which this verified statement is directed.

Chi-Tang Ho Dec. 23. 2004

*Chi-Tang Ho*

*Date*

Shengmin Sang

*Shengmin Sang*

*Date*

12/23/2004

Continuing from page 9

TPA ( ) preparation

25  $\mu$ l test compound = 0.5  $\mu$ mol for each compound

TPA

1. Thae flavin

2. Thae flavin-3'-gallate

3. Thae flavin-3'-gallate

4. Thae flavin-3'-2'-digallate

5. EGCG

6. Acetone

10:30 AM

Kitten ~ Thae flavin

10:50 AM

TPA

2:50 PM

Kitten killed

Acetone

TPA

Thae flavin (0.5  $\mu$ mol)Thae flavin-3'-gallate (0.5  $\mu$ mol)Thae flavin-3'-gallate (0.5  $\mu$ mol)Thae flavin-3'-2'-digallate (0.5  $\mu$ mol)EGCG (0.5  $\mu$ mol)OD + feces collected at  
age of 23 days old  
30 days old at

The compound in 20  $\mu$ l acetone was applied topically  
on both sides 20 min before TPA application.

/ Inhibition

1	74.4/10 ears	7.44	0	-	7.44 $\pm$ 0.18 (0.08)	P = 0.08
2	117.0/10 ears	11.70	4.26	-	11.70 $\pm$ 3.05 (1.38)	
3	79.6/10 ears	7.96	0.46	89.2%	7.90 $\pm$ 0.85 (0.42)	P = 0.015
4	78.6/10 ears	7.86	0.36	91.5%	7.86 $\pm$ 0.53 (0.24)	P = 0.013
5	74.4/10 ears	7.44	0	100.0%	7.44 $\pm$ 0.40 (0.18)	P = 0.008
6	70.3/10 ears	7.03	-0.41	100.0%	7.03 $\pm$ 0.40 (0.18)	P = 0.03
7	87.9/10 ears	8.79	1.35	68.3%	8.79 $\pm$ 1.20 (0.54)	P = 0.043

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PROJECT

Exp HR-1

Effect of a single dose of theoflavin on

Notebook No.

TPA - induced edema converted From Page 201

A single treatment

1. Acetone	$74.4/10 \text{ ems} + 198. = 272.4 \text{ mg}$
2. TPA	$117.0/10 \text{ ems} + 341.6 = 458.6 \text{ g}$
3. TPA + theoflavin	$79.6/10 \text{ ems} + 305.5 = 385.1 \text{ g}$
4. TPA + theoflavin-3-G	$78.6/10 \text{ ems} + 304.1 = 382.7 \text{ g}$
5. TPA + theoflavin-3'-G	$74.4/10 \text{ ems} + 347.6 = 422.0 \text{ g}$
6. TPA + theoflavin-3'-di-G	$70.3/10 \text{ ems} + 297.9 = 368.2 \text{ mg}$
7. EGCG	

pool 2 ears weight

1. Acetone	14.6 (7.34), 15.1 (7.55g), 14.4 (7.20g), 15.1 (7.55g), 15.4 (7.70g)
2. TPA	27.0 (13.5g), 33.5 (16.75), 17.4 (8.7), 19.2 (9.6), 18.6 (9.3)
3. TPA + theoflavin	19.3 (9.65), 14.3 (7.15), 15.4 (7.70), 14.1 (7.05), 16.7 (8.35)
4. TPA + theoflavin-3-G	17.6 (8.8), 14.8 (7.4), 15.4 (7.70), 14.7 (7.35), 16.1 (8.05)
5. TPA + theoflavin-3'-G	15.4 (7.70), 14.8 (7.4), 15.9 (7.95), 13.6 (6.8), 14.1 (7.05)
6. TPA + theoflavin-3'-di-G	13.8 (6.90), 15.7 (7.85), 13.9 (6.90), 14.1 (7.05), 13.4 (6.70)
7. EGCG	17.7 (8.85), 22.1 (11.05), 16.5 (8.25), 15.0 (7.50), 16.9 (8.45)

n=5

 $\sqrt{5} = 2.236$  $E = \frac{D}{\sqrt{5}}$ 

IL-6 (pg/ml)

1. Acetone	$7.46 \pm 0.18$ (0.08)	$0.88 \pm 0.07$ (0.05)	n=2
2. TPA + theoflavin	$4.11 \pm 0.57$ (1.38)	$3.05 \pm 0.05$ (0.03)	2.17
3. TPA + theoflavin-3-G	$0.33 \pm 0.79$ (0.95)	$2.48 \pm 0.16$ (0.11)	1.60 - 26.3
4. TPA + theoflavin-3'-G	$0.40 \pm 0.86$ (0.53)	$1.76 \pm 0.05$ (0.03)	0.88 - 59.4
5. TPA + theoflavin-3'-di-G	$0.74 \pm 0.40$ (0.18)	$1.08 \pm 0.03$ (0.02)	0.20 - 90.8
6. TPA + theoflavin-3'-di-G	$0.708 \pm 0.40$ (0.18)	$1.52 \pm 0.14$ (0.10)	0.64 - 70.5
7. EGCG	$1.36 \pm 0.82$ (1.20)	$2.10 \pm 0.06$ (0.04)	1.22 - 43.8

page 9.

Theoflavin and its derivatives stock solutions  
were diluted 1:1

3. 300  $\mu$ l acetone + 300  $\mu$ l theoflavin
4. 300  $\mu$ l acetone + 300  $\mu$ l theoflavin-3-G
5. 300  $\mu$ l acetone + 300  $\mu$ l theoflavin-3'-G
6. 300  $\mu$ l acetone + 300  $\mu$ l theoflavin-3'-di-G
7. 300  $\mu$ l acetone + 300  $\mu$ l EGCG

\* 20  $\mu$ l = 0.4  $\mu$ mol12.5  $\mu$ l = 0.25  $\mu$ mol

Continued on Page

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10.2 = 0.20  $\mu$ mol

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PROJECT

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Continued From Page \_\_\_\_\_

HR-4 (page 26)

1	Acetone + Acetone	7.09 ± 0.28 (0.11)	0	0	Inhibition
2	Acetone + TPA (0.3 μmol)	10.01 ± 1.28 (0.52)	2.92		
3	Nobelator <sup>10-15 μmol</sup> + TPA (0.3 μmol)	11.20 ± 1.18 (0.48)	4.11	↑40.8%	
4	Nobelator (0.5 μmol) + TPA (0.3 μmol)	8.94 ± 0.64 (0.26)	1.85	36.6%	
5	Dimenacetone (0.25 μmol) + TPA (0.3 μmol)	8.69 ± 1.23 (0.50)	1.60	45.2%	
6	Dimenacetone (0.50 μmol) + TPA (0.3 μmol)	7.99 ± 0.29 (0.12)	0.90	69.2%	
7	Glycolic acid (0.5 μmol) + TPA (0.3 μmol)	9.85 ± 1.53 (0.47)	2.76	5.5%	
8	Glycolic acid (2.0 μmol) + TPA (0.3 μmol)	11.84 ± 0.94 (0.38)	4.75	↑62.7%	

Female C57BL/6J (28-30 days old; 6 mice/group) were treated topically with 20 μl acetone or test compound in acetone 20 μl topically application of TPA (0.3 μmol) five a day for 3 days and half. The mice were killed at 5 hr after the last dose of TPA treatment and punches were taken and weighed. Data are expressed as the mean ± S.E.

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## PROJECT \_\_\_\_\_

Continued From Page \_\_\_\_\_

# HR-5 Time Course of TPA - Induced Inflammation and Cytokine Protein Expression

TPA (1.6  $\mu$ M)

1	0	No TPA	(Tuesday, killed)
2	3	1:30 p.m. $\rightarrow$ 4:30 p.m.	(Tuesday)
3	5	11:35 p.m. $\rightarrow$ 4:35 p.m.	(Tuesday)
4	8	8:40 p.m. $\rightarrow$ 4:40 p.m.	(Tuesday)
5	16	5:10 p.m. $\rightarrow$ 9:10 a.m.	(Wednesday)
6	24	9:10 a.m. $\rightarrow$ 9:10 a.m.	(Wed $\rightarrow$ Thurs)
7	48	9:10 $\rightarrow$ 9:10 a.m.	(Tuesday $\rightarrow$ Thursday)
8	72	9:15 $\rightarrow$ 9:35 a.m.	(Monday $\rightarrow$ Thursday)

(5 mice per group)

Female CD-1 mice (9-10 weeks old) were treated topically with TPA (1.6  $\mu$ M) in 20  $\mu$ l acetone once. The mice were killed at 0, 3, 5, 8, 16, 24, 48 and 72 hours after TPA treatment. Ear punches were taken and weighed (both ears). All ear samples were stored in a  $-80^{\circ}\text{C}$  for cytokine assay.

1	0 hrs:	17.1 (8.55), 17.5 (8.75), 16.2 (8.10), 17.1 (8.55), 16.2 (8.10)	
		total 10 ears, 86.3	other total 28.80
2	3 hrs:	16.8 (8.40), 14.9 (7.45), 16.9 (8.45), 14.3 (7.15), 17.4 (8.70)	
		total 10 ears, 80.8	total other 38.20
3	5 hrs:	28.2 (14.1), 21.0 (10.5), 26.4 (13.2), 27.8 (13.9), 31.8 (15.9)	
		total 10 ears, 13.56	total other 53.53
4	8 hrs:	37.4 (18.7), 28.5 (14.25), 32.9 (16.45), 36.7 (18.35), 36.9 (18.45)	
		total 10 ears, 17.58	total other 69.31
5	16 hrs:	24.3 (12.15), 25.1 (12.55), 32.0 (16.00), 19.3 (9.65), 28.1 (14.05)	
		total 10 ears, 13.19	others 47.74
6	24 hrs:	22.5 (11.25), 19.1 (9.55), 22.9 (11.45), 17.8 (8.9), 24.8 (12.4)	
		total 10 ears, 10.78	others 45.12
7	48 hrs:	16.3 (8.15), 16.2 (8.10), 17.5 (8.75), 16.7 (8.35), 15.9 (8.45)	
		total 10 ears, 82.7	other 36.76
8	72 hrs:	18.9 (9.45), 17.6 (8.80), 17.5 (8.75), 18.3 (9.15), 18.6 (9.30)	
		total 90.7	others 35.75

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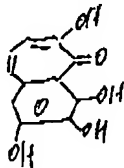
hrs after TPA

1	0	$8.41 \pm 0.26$ (0.12)	(8.63)	100%
2	3	$8.03 \pm 0.61$ (0.27)	(8.08)	95.5%
3	5	$13.52 \pm 1.75$ (0.78)	(13.56)	160.8%
4	8	$17.24 \pm 1.69$ (0.76)	(17.58)	205%
5	16	$12.88 \pm 2.11$ (0.94)	(13.14)	153.2%
6	24	$10.71 \pm 1.29$ (0.58)	(10.78)	127.3%
7	48	$8.36 \pm 0.23$ (0.10)	(8.27)	99.4%
8	72	$9.09 \pm 0.27$ (0.12)	(9.07)	108.1%

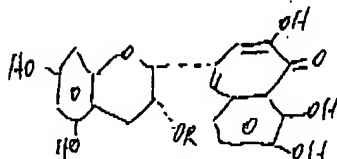
TPA (1.6 nmol) bath ears

See pages 38 and 39

11. GaCa: F.W. 220, solvent, Acetone, Pyridone. Amount: 64 mg



12, and 13



12: EGCGa; R=H, F.W. 384, solvent, Acetone, Pyridone. Amount: 51

13: EGCGa; R=gallate, F.W. 536, solvent: Acetone, Pyridone, Amount: 51

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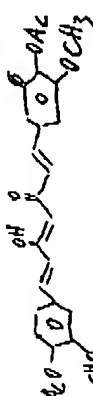
## PROJECT

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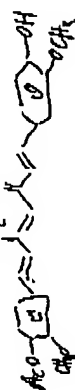
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1. Total: 13 Samples,  
 FCM: SHENGENIN SANG

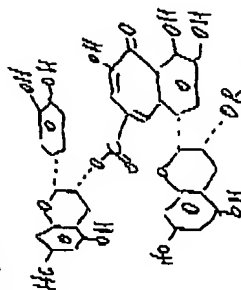
1. Cu-1: F.W.: 452, solvent: CHCl<sub>3</sub>, Acetone, Amount: 50mg



2. Cu-2: F.W.: 452, solvent: CHCl<sub>3</sub>, Acetone, Amount: 50mg



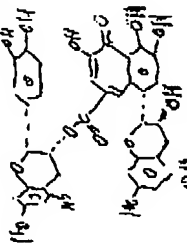
3 and 4:



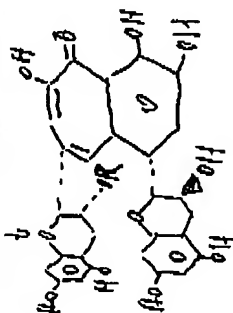
3: ~~ECG~~ ECGa; R = R gallate, F.W.: 552, solvent: Acetone, MeOH, Amount: 50mg

4: EECG; R = H, F.W.: 700, solvent: Acetone, MeOH, Amount: 50mg

5: CECG; F.W.: 700, solvent: Acetone, MeOH, Amount: 50mg

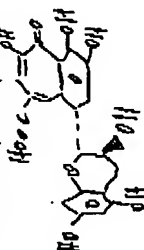


6. CEGC; F.W.: 564, solvent: Acetone, MeOH, Amount: 53mg

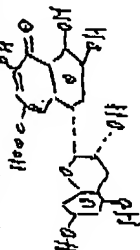


7. CEGCg; R = gallate, F.W.: 716, solvent: Acetone, MeOH, Amount: 51mg

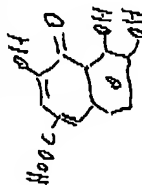
8. CGA; F.W.: 428, solvent: Acetone, MeOH, Amount: 43mg



9. ECGA; F.W.: 428, solvent: Acetone, MeOH, Amount: 51mg



10. GKa; F.W.: 248, solvent: Acetone, MeOH, Amount: 61mg



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Name	mole wt.	Amount	20 $\mu$ l = 0.5 $\mu$ mol	20 $\mu$ l = 1 $\mu$ mol
1. Cu-1	452	50 mg	11.3 mg/ml	4.42 ml
2. Cu-2	452	50 mg	11.3 mg/ml	2.21 ml
3. EC6di	852	50 mg	21.3 mg/ml	2.21 ml
4. ECECG	700	50 mg	17.5 mg/ml	1.175 ml
5. CECG	700	50 mg	17.5 mg/ml	1.243 ml
6. CEGC	564	53 mg	14.1 mg/ml	1.429 ml
7. CEGCG	716	51 mg	17.9 mg/ml	1.879 ml
8. CGA	428	43 mg	10.7 mg/ml	1.425 ml
9. ECGA	428	51 mg	10.7 mg/ml	2.01 ml
10. GACA	248	61 mg	6.2 mg/ml	2.335 ml
11. GACA	220	64 mg	5.5 mg/ml	4.919 ml
12. ECGCA	384	51 mg	12.75 mg/ml	5.815 ml
13. ECGCA	536	51 mg	3.4 mg/ml	2.00 ml
14. Caxemum	368	51 mg	9.2 mg/ml	1.903 ml
# 10	20 $\mu$ l = 2.0 $\mu$ mol		2.455 ml	
	= 1.0 $\mu$ mol		4.919 ml	
	= 0.5 $\mu$ mol		9.838 ml	
# 11	20 $\mu$ l = 2.0 $\mu$ mol		11.63 ml	2.908 ml
	= 1.0 $\mu$ mol		5.815 ml	
	= 0.5 $\mu$ mol		11.63 ml	
1-7	20 $\mu$ l = 1 $\mu$ mol			
8-13	20 $\mu$ l = 0.5 $\mu$ mol			

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PROJECT \_\_\_\_\_

Notebook No. \_\_\_\_\_

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### Acetylation and Methylation of Curcumin and EGCG

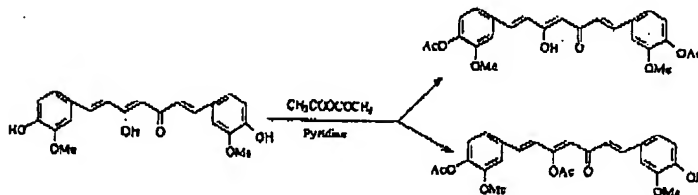
#### Rational and Objectives

Curcumin and EGCG, the know component in turmeric and tea, respectively, are important chemopreventive agents. It is know that these two compounds have poor bioavailability due to their high polarity. We will synthesize the acetylated and methylated derivatives of curcumin and EGCG in the hope to improve their bioavailability. 100-200 mg samples of each derivative of curcumin and EGCG will first be prepared to give to Drs. M. T. Huang for bioactivity study and also to Dr. C.S. Yang for bioactivity study.

#### 1. Acetylation of Curcumin

We have successfully synthesized two diacetylated isomers of curcumin. 500 mg of curcumin was reacted with 0.3 mL acetyl anhydride in pyridine (3 mL) at room temperature for 6 hr. After evaporation of the solvent *in vacuo*, the residue was applied to silica gel column eluted with hexane-chloroform-methanol (2.5:2:0.2) solvent system to give 120 mg compound 1, 200 mg compound 2, and 200 mg curcumin. Both compound 1 and 2 are diacetylated isomers of curcumin. The structures of these two compounds have been established by mass spectrometry and NMR spectrometry.

We will synthesize the mono- and tri- acetylated curcumin by controlling the amount of acetyl anhydride, reaction time, and basicity of solvent.



#### 2. Acetylation of EGCG

Using the same method we used for the preparation of the acetylated curcumin, we will be able to synthesize different acetylated EGCG. We will use Sephadex LH-20 and RP C-18 to isolate these reaction products. The structure of synthesized compounds will be identified by mass spectrometry and NMR spectrometry.

#### 3. Methylation of EGCG

EGCG will be mixed with methyl iodide and  $K_2CO_3$  in aqueous acetone. We will control the degree of methylation in EGCG by changing the ratio of EGCG and methyl iodide, and also the reaction time. The products will be isolated by the combination of Sephadex LH-20 column and RP C-18 column chromatography. This method has been successfully used by Dr. Meng as described in his thesis (Meng, X., Ph.D. Thesis, Rutgers University, 2002).

#### 4. Methylation of Curcumin

The method for the methylation of EGCG will be applied to the methylation of curcumin. Again, the synthesized derivatives will be structurally identified by mass spectrometry and NMR spectrometry.

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1. Acetone + Acetone 7.40 ± 0.15 (0.07) 0 7.47

2. Acetone + TPA (1 mmol) 15.91 ± 1.15 (0.51) 8.47 (88.84) 16.5

3. Acetone + TPA (1 mmol) 8.75 ± 0.71 (0.32) 0.91 (89.27) 8.35

4. Acetone + TPA (1 mmol) 8.52 ± 0.59 (0.24) 0.08 (87.26) 8.60

5. Acetone + TPA (1 mmol) 8.65 ± 1.43 (0.64) 1.24 (85.74) 8.73

6. Acetone + TPA (1 mmol) 10.58 ± 1.63 (0.73) 3.14 (82.91) 10.41

7. Acetone + TPA (1 mmol) 8.42 ± 0.93 (0.44) 0.98 (88.47) 8.57

8. Acetone + TPA (1 mmol) 9.28 ± 0.82 (0.37) 1.24 (78.37) 9.24

Inhibitory Effect of Theaflavin's Derivatives  
on 12-O-Tetradecanoylphorbol-13 acetate (TPA)-induced  
Edema of Mouse Ear

Treatment	Average weight of ear punches (mg) (Mean ± SD)	Percent inhibition
1. Acetone + Acetone	7.44 ± 0.17	-
2. Acetone + TPA (1 mmol)	15.91 ± 0.51	-
3. EGCG (0.5 μmol) + TPA (1 mmol)	8.35 ± 0.32*	89.2%
4. EGCG (0.3 μmol) + TPA (1 mmol)	8.52 ± 0.26*	97.2%
5. CEQO (0.5 μmol) + TPA (1 mmol)	8.65 ± 0.64	85.7%
6. CEQO (0.3 μmol) + TPA (1 mmol)	10.28 ± 0.73*	62.9%
7. CEQO (0.5 μmol) + TPA (1 mmol)	9.42 ± 0.72*	85.4%
8. CGA (0.5 μmol) + TPA (1 mmol)	9.28 ± 0.37*	78.3%

Both ears of female CD-1 mice (5 mice per group; 35 days old) were treated topically with 20 μl acetone, or test compound in 20 μl acetone at 20 min prior to topical treatment of 20 μl acetone or TPA (1 mmol) in 20 μl acetone. Five hours later, the mice were killed by cervical dislocation and ear punches (6-mm in diameter) were taken and weighed.

\*Statistically different from the second TPA treated group (P < 0.05) as determined by the Student's t-test.

1. Ethyl Acetate fraction Cranberry

1. Ethyl Acetate fraction Cranberry (Kagoshima)

(2) Butanol fraction Cranberry (Kagoshima)

(2) Butanol fraction Cranberry (dried)

(3) Aqueous fraction Cranberry (dried)

Lot No. 2145 Lot No. 2157 stored -20°C

17/12/11/12 - 50 mg/kg body weight of acid (98%) 25 mg

11/12/11/12 - 50 mg/kg body weight of acid (98%) 25 mg

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metreya, Inc

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PROJECT

M-T Huang

Notebook No. \_\_\_\_\_

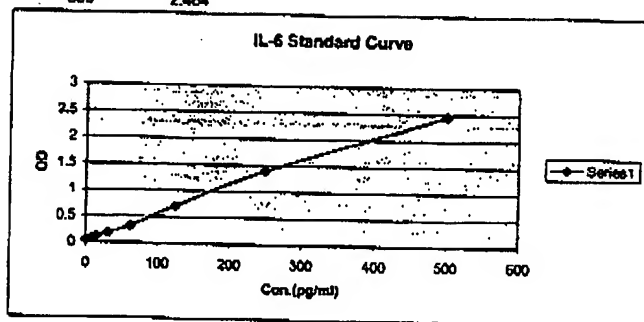
IL-6 Standard Curve  
con.(pg/ml) OD

0	0.051
7.8	0.077
15.6	0.123
31.2	0.188
62.5	0.327
125	0.71
250	1.397
500	2.464

- 1 tissue con. 100mg/ml
- 2 Dilution (1:8)
- 3 100ul sample for assay.
- 4 OD used wavelength 450nm.

Exp HR-5

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HR-5 Time Course of TPA Induced Inflammation (1:8)

# of group	Group	OD	Con. (pg/ml)			
1	0 hour (no TPA)	0.107	13.57	13.57	13.57	0.07 ± 0
2	3 hours (TPA)	0.172	28.55	28.55	28.55	1.71 ± 0
3	5 hours (TPA)	0.437	83.52	87.35	95.69	1.71 ± 0
4	8 hours (TPA)	0.477	91.17	87.345	55.14	5.47 ± 0.23 (0.16)
5	16 hours (TPA)	0.547	85.3	95.685	35.54	5.78 ± 0.04 (0.03)
6	24 hours (TPA)	0.294	58.19	55.14	7.95	3.37 ± 0.07 (0.05)
7	48 hours (TPA)	0.176	29.21	35.535	2.24	2.13 ± 0.58 (0.27)
8	72 hours (TPA)	0.178	29.54	22.825	1.77	1.37 ± 0.40 (0.28)
9	84 hours (TPA)	0.079	8	7.95	0.47	0.48 ± 0 (0)

Dr. Bob Rosen,

W: Bioassay results?

1

Delivered-To: bobrosen@aesop.rutgers.edu  
 Date: Mon, 1  
 From: "Dr. Bob Rosen" <rosen@AESOP.RUTGERS.EDU>  
 Subject: FW: Bioassay results?  
 To: nichuang@aesop.rutgers.edu  
 Cc: vorsa@AESOP.RUTGERS.EDU  
 Reply-to: rosen@AESOP.RUTGERS.EDU  
 Importance: Normal  
 X-Priority: 3 (Normal)  
 Status:

MT

Ch-Tang sent over some samples that Nick Vorsa extracted. These were from blueberry and/or cranberry.

Did you have a chance to do a mouse ear test yet to see which extract best reduced inflammation?

Nick needs any simple assay to see which extract is best to continue with his isolations

Thanks

Bob Rosen

-----Original Message-----

From: Nichol Vorsa [mailto:vorsa@AESOP.RUTGERS.EDU]  
 Sent: Wednesday,  
 To: Dr. Bob Rosen  
 Subject: Bioassay results?

Bob:

I hope you are having a good summer. Iina and I are wondering how the bioassay(s) went and when might the results be available?

regards,

Nick

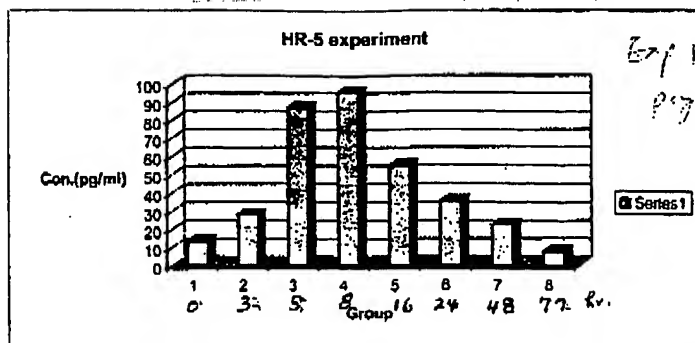
Continued on Page

By

Huang

AC

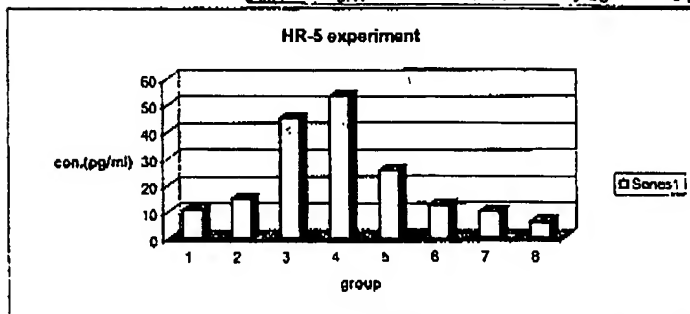
Date



Exp HR-5  
Page 29-3

HR-5 Time Course of TPA Induced Inflammation (1:12) 8-33-2016 n=4 P8/mg

# of group	Group	OD	Con. (pg/ml)	Mean	SD	Mean	SD	Mean	SD
0	1 0 hour (no TPA)	0.12	15.22	10.8	0.81	10.8	1.83	1.14 ± 0.49 (0.30)	
		0.083	6.38	15.03	1.71	15.03	1.83	1.14 ± 0.49 (0.30)	
3	2 3 hours (TPA)	0.121	15.22	15.03	1.71	15.03	1.83	1.14 ± 0.49 (0.30)	
		0.117	14.84	15.03	1.71	15.03	1.83	1.14 ± 0.49 (0.30)	
5	3 4 hours (TPA)	0.265	43.98	45.06	5.01	25.73	5.28	1.76 ± 0.65 (0.03)	
		0.278	46.14	45.06	5.01	25.73	5.28	1.76 ± 0.65 (0.03)	
8	4 5 hours (TPA)	0.289	55.24	53.425	5.78	10.13	6.63	5.33 ± 0.21 (0.11)	
		0.27	51.61	53.425	5.78	10.13	6.63	5.33 ± 0.21 (0.11)	
16	5 6 hours (TPA)	0.182	26.39	25.725	3.57	3.17	3.17	6.08 ± 0.37 (0.14)	
		0.151	25.06	25.725	3.57	3.17	3.17	6.08 ± 0.37 (0.14)	
24	6 7 hours (TPA)	0.078	7.8	12.64	1.75	0.95	0.95	3.20 ± 0.13 (0.07)	
		0.137	17.38	12.64	1.75	0.95	0.95	3.20 ± 0.13 (0.07)	
48	7 8 hours (TPA)	0.084	9.52	10.13	1.77	1.14	1.14	1.83 ± 0.57 (0.29)	
		0.106	10.74	10.13	1.77	1.14	1.14	1.83 ± 0.57 (0.29)	
72	8 9 hours (TPA)	0.087	6.79	6.13	0.44	0.82	0.82	1.29 ± 0.70 (0.15)	
		0.054	5.47	6.13	0.44	0.82	0.82	1.29 ± 0.70 (0.15)	



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PR

- 1 Issue con. 100mg/ml  
2 Dilution (1:5, 1:10)  
3 50ul sample for assay  
4 OD used wavelength 450nm

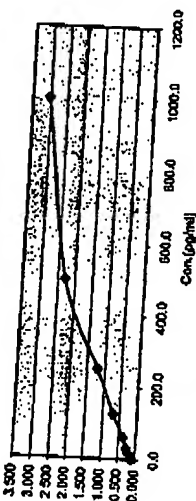
Exp HA-5

page 29-30

N-1B Standard Curve

Con. (pg/ml)	OD
0.0	0.121
15.6	0.188
31.2	0.272
62.5	0.371
125.0	0.689
250.0	1.209
500.0	2.287
1000	3.028

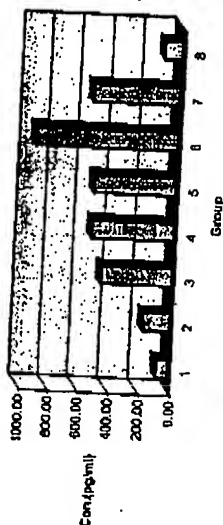
N-1B Standard Curve



HR-5 Time Course of TPA Induced Inflammation (1:5) 200ug/4

# of group	10 hour (no TPA)	OD	Con. (pg/ml)
1	0.451	75.98	78.74
2	0.46	77.48	78.74
3	0.98	171.67	172.475
4	0.988	173.28	172.475
5	2.127	465.02	459.01
6	2.072	453	453.83
7	2.48	542.2	532.36
8	2.39	522.52	511.00
9	2.391	522.74	514.99
10	2.485	538.92	530.83
11	2.823	931.98	834.465
12	2.838	930.94	916.80
13	2.568	561	561
14	2.589	561	561
15	0.837	113.81	114.985
16	0.648	118.06	51.9
17	0.107	0	0
18	0.097	0	0

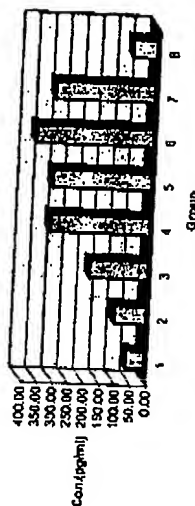
HR-5 experiment



HR-5 Time Course of TPA Induced Inflammation (1:10)

# of group	10 hour (no TPA)	OD	Con. (pg/ml)
1	0.372	82.87	59.47
2	0.334	58.27	59.47
3	0.575	102.83	102.83
4	0.575	102.83	102.83
5	1.082	118.09	185.39
6	1.222	252.89	314.62
7	1.472	304.38	318.985
8	1.584	329.61	315.55
9	1.543	319.07	314.62
10	1.5	310.17	310.17
11	1.737	378.76	371.885
12	1.665	394.01	371.885
13	1.473	304.59	315.55
14	1.578	326.51	326.51
15	0.43	72.44	74.985
16	0.46	77.48	77.48
17	0.118	0	0
18	0.125	0	0

HR-5 Experiment



M-T Huang

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Date

PROJECT

Notebook No.

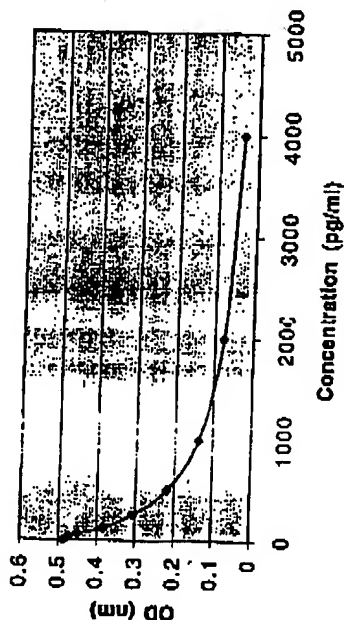
- 1) Dilution 1:100  
2) 50 ul sample for assay  
3) OD used wavelength 420 nm

0.4865  
0.4745  
0.451  
0.3825  
0.3065  
0.218  
0.138  
0.078  
0.036

Stat-PGE2 Standard Curve  
Concentration (pg/ml)

0  
31.25  
62.5  
125  
250  
500  
1000  
2000  
4000

Stat-PGE2 Standard Curve



Concentration (pg/ml)  
NOT ON GRAPH  
0.501  
0.33  
0.218  
0.242  
0.303  
0.163  
0.508  
0.141  
0.151

Sample (1:100 Dilution)  
Buffer

- 1) 0 hrs - No TPA  
2) 3 hrs  
3) 5 hrs  
4) 8 hrs  
5) 16 hrs  
6) 24 hrs  
7) 48 hrs  
8) 72 hrs

Blank = 0.084

pg/ml

DATA	Raw Data	Minus Blank	Average
B0	0.558	0.472	0.4865
B1	0.585	0.501	0.4745
F1	0.534	0.47	0.451
F2	0.563	0.478	0.3825
F3	0.512	0.428	0.3065
F4	0.558	0.474	0.218
F5	0.453	0.369	0.138
F6	0.48	0.399	0.078
F7	0.385	0.301	0.036
F8	0.396	0.312	0.218
F9	0.297	0.213	0.138
F10	0.307	0.223	0.076
F11	0.213	0.129	0.036
F12	0.231	0.147	0.501
F13	0.184	0.08	0.242
F14	0.156	0.072	0.303
F15	0.121	0.037	0.242
F16	0.118	0.035	0.163
F17	0.562	0.478	0.501
F18	0.605	0.521	0.242
F19	0.598	0.504	0.242
F20	0.228	0.142	0.133
F21	0.211	0.127	0.133
F22	0.215	0.131	0.133
F23	0.312	0.228	0.218
F24	0.302	0.218	0.218
F25	0.286	0.202	0.202
F26	0.325	0.241	0.242
F27	0.318	0.234	0.234
F28	0.336	0.252	0.252
F29	0.404	0.32	0.303
F30	0.376	0.292	0.292
F31	0.381	0.287	0.287
F32	0.258	0.174	0.163
F33	0.244	0.156	0.156
F34	0.277	0.193	0.193
F35	0.344	0.28	0.28
F36	0.248	0.164	0.164
F37	0.23	0.146	0.141
F38	0.225	0.141	0.141
F39	0.221	0.137	0.137
F40	0.236	0.152	0.152
F41	0.228	0.144	0.144
F42	0.242	0.158	0.158

1:100 Dilution

Concentration (pg/ml)  
pg/ml

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

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Date

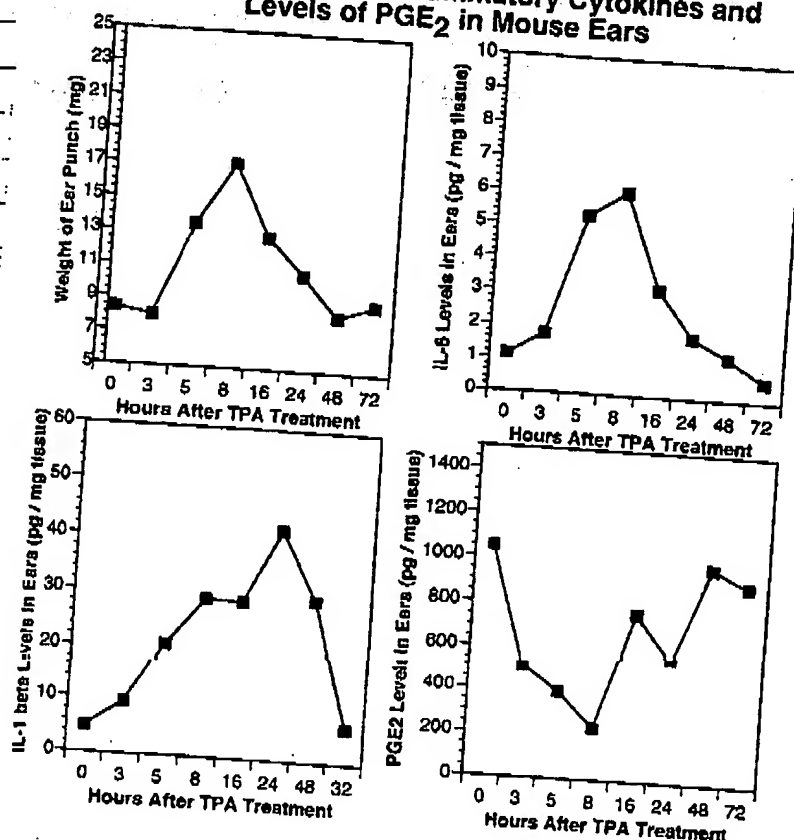
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PROJECTok No. \_\_\_\_\_  
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HR-5 (p 29 + 30)

Time Course of TPA-induced Ear Edema,  
Production of Pro-inflammatory Cytokines and  
Levels of PGE<sub>2</sub> in Mouse EarsExp HR-5 Time course of TPA-induce ear edema and increases in  
expression of pro-inflammatory cytokine genes in CD-1 mice

Hours after TPA	IL-1 beta (pg / mg)	IL-6 (pg / mg)	TNF alpha (pg / mg)	PGE <sub>2</sub> (pg / mg)	LTB <sub>4</sub> (pg / mg)	Edema (mg/punch)
0	4.89±0.54	1.14±0.30		1053.33±23.73	10.30	8.41±0.12
3	9.45±0.42	1.76±0.03		506.67±19.05	8.45	8.03±0.27
5	20.75±2.62	5.33±0.11		396.67±11.86	8.30	13.50±0.78
8	29.16±1.36	6.08±0.14		236.67±15.15	6.25	17.24±0.76
16	29.01±0.96	3.20±0.07		773.33±62.78	8.15	12.88±0.94
24	41.96±2.40	1.83±0.29		565.00±100.19	14.00	10.71±0.58
48	29.80±0.96	1.29±0.15		990.00±4.71	16.67	8.36±0.10
72	6.49±0.37	0.61±0.07		913.33±33.45	8.45	9.09±0.12

Female CD-1 mice (9-10 weeks old; 5 mice per group) were treated topically with acetone or TPA (1.6 nmol) in 20 µl acetone. The mice were killed by cervical dislocation At 3, 5, 8, 16, 24, 48, and 48 hours after TPA treatment. Ear punches (6 mm- in diameter) were taken and weighed. The ear samples were stored in a - 80 °C freezer for cytokine assays.

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PROJECT

AR-9 (Effect of Black Tea on TPA study of mouse ear edema)

continued from Page 14

		mean $\pm$ SE	% Inhibition
1	Acetone (20 $\mu$ l)	7.17 $\pm$ 0.41 (0.18)	- $p = 4.4 \times 10^{-6}$
2	TPA (1 nmol) = 20 $\mu$ l	15.85 $\pm$ 1.91 (0.84)	-
3	C8 (20 $\mu$ l = 0.5 nmol) + TPA (1 nmol)	8.95 $\pm$ 0.97 (0.43)	79.5% $p = 4.6 \times 10^{-5}$
4	C9 ( " ) + TPA (1 nmol)	9.39 $\pm$ 1.39 (0.62)	74.4% $p = 1.42 \times 10^{-5}$
5	C11 ( " ) + TPA (1 nmol)	8.61 $\pm$ 1.19 (0.54)	83.4% $p = 4.15 \times 10^{-5}$
6	C12 ( " ) + TPA (1 nmol)	7.86 $\pm$ 0.41 (0.18)	92.1% $p = 8.23 \times 10^{-5}$
7	C13 ( " ) + TPA (1 nmol)	7.49 $\pm$ 0.76 (0.32)	96.3% $p = 5.38 \times 10^{-5}$
8	thai flavon ( " ) + TPA (1 nmol)	8.23 $\pm$ 0.90 (0.40)	87.8% $p = 2.05 \times 10^{-5}$

Female C10-1 (6 weeks old)

1	Acetone	13.8 (6.4), 14.5 (7.25), 14.4 (7.20), 15.2 (7.60), 14.8 (7.40)	total 10 ears, 7.12 mg/ear, other 2+3=
2	TPA	15.45 (8.9), 25.0 (12.5), 36.3 (18.15), 34.2 (19.10), 32.1 (16.05)	total 10 ears, 15.86 mg/ear, other 494 mg
3	C8	20.7 (10.35), 19.5 (9.75), 15.7 (7.85), 17.5 (8.75), 16.1 (8.05)	total 10 ears, 90.3 mg/ear, total other 402.4 mg
4	C9	17.2 (8.60), 19.7 (9.85), 23.8 (11.90), 17.3 (8.65), 15.9 (7.95)	total 10 ears, 92.2 mg/ear, other 334.7 mg
5	C11	19.0 (9.50), 13.4 (6.70), 16.4 (8.20), 22.4 (11.2), 16.9 (8.45)	total 10 ears, 8.68 mg/ear, other 367.1 mg
6	C12	16.2 (8.1), 15.0 (7.5), 15.2 (7.6), 17.1 (8.55), 15.1 (7.55)	total 10 ears, 7.87 mg/ear, other 367.1 mg
7	C13	14.3 (7.15), 15.7 (7.85), 15.3 (7.65), 14.5 (7.25), 15.1 (7.55)	total 10 ears, 7.42 mg/ear, total 305.9 mg
8	Thaiflavin	16.8 (8.4), 19.0 (9.5), 14.0 (7.0), 17.6 (8.8), 14.9 (7.45)	total 10 ears, 8.43 mg/ear, 331.7 mg

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**Inhibitory Effect of Theaflavin's Derivatives  
on 12-O-Tetradecanoylphorbol-13 acetone (TPA)-induced  
Edema of Mouse Ear**

Treatment	Average weight of ear punches (mg) (Mean $\pm$ SE)	Percent inhibition
1. Acetone + Acetone	7.17 $\pm$ 0.18*	-
13 Acetone + TPA (1 nmol)	15.85 $\pm$ 0.86	-
8. CGA (0.5 $\mu$ mol) + TPA (1 nmol)	8.95 $\pm$ 0.43*	79.5%
9. EGCGA (0.5 $\mu$ mol) + TPA (1 nmol)	9.39 $\pm$ 0.43*	74.4%
11. gaCa (0.5 $\mu$ mol) + TPA (1 nmol)	8.61 $\pm$ 0.54*	83.3%
12. EGCCa (0.5 $\mu$ mol) + TPA (1 nmol)	7.86 $\pm$ 0.18*	92.1%
13. EGCGCa (0.5 $\mu$ mol) + TPA (1 nmol)	7.49 $\pm$ 0.12*	96.3%
14 Theaflavin (0.5 $\mu$ mol) + TPA (1 nmol)	8.23 $\pm$ 0.40*	87.8%

Both ears of female CD-1 mice (5 mice per group; 35 days old) were treated topically with 20  $\mu$ l acetone, or test compound in 20  $\mu$ l acetone at 20 min prior to topical treatment of 20  $\mu$ l acetone or TPA (1 nmol) in 20  $\mu$ l acetone. Five hours later, the mice were killed by cervical dislocation and ear punches (6-mm in diameter) were taken and weighed.

\*Statistically different from the second TPA treated group ( $P < 0.05$ ) as determined by the Student's  $t$  test.

HR-10

**Inhibitory effect of curcumin and curcumin acetate on TPA-induced  
edema of mouse ear**

Treatment	Number of mice per group	Weight of ear punch (mg)	Percent inhibition
1. Acetone	5	7.61 $\pm$ 0.35*	-
2 TPA (1 nmol)	5	11.94 $\pm$ 0.90	-
3. Curcumin acetate-1 (0.25 $\mu$ mol) + TPA (1 nmol)	5	9.24 $\pm$ 0.29*	62.4%
4. Curcumin acetate-1 (0.75 $\mu$ mol) + TPA (1 nmol)	5	7.39 $\pm$ 0.20*	100.0%
5. Curcumin acetate-2 (0.25 $\mu$ mol) + TPA (1 nmol)	5	7.75 $\pm$ 0.15*	96.8%
6. Curcumin acetate-2 (0.75 $\mu$ mol) + TPA (1 nmol)	5	7.45 $\pm$ 0.19*	100.0%
7. Curcumin (0.25 $\mu$ mol) + TPA (1 nmol)	5	8.58 $\pm$ 0.30*	77.6%
8. Curcumin (0.75 $\mu$ mol) + TPA (1 nmol)	5	7.92 $\pm$ 0.30*	92.8%

Female CD-1 mice (5 weeks old; 5 mice per group) were treated topically with 20  $\mu$ l acetone or test compound in 20  $\mu$ l acetone at 20 minutes before topical application of 20  $\mu$ l acetone or TPA (1 nmol) in 20  $\mu$ l acetone. Five hours later, all mice were killed by cervical dislocation. Ear punches (6-mm in diameter) were taken and weighed. Data are expressed as the mean  $\pm$  SE.

\*Statistically different from group 2 TPA alone ( $P < 0.05$ ) determined by the Student's  $t$  test.

Signature \_\_\_\_\_  
Date \_\_\_\_\_

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